Adaptive zones shape the magnitude of premating reproductive isolation in *Timema* stick insects

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Abstract

Simpson's fossil-record inspired model of 'adaptive zones' proposes that evolution is dominated by small fluctuations within adaptive zones, occasionally punctuated by larger shifts between zones. This model can help explain why the process of population divergence often results in weak or moderate reproductive isolation (RI), rather than strong RI and distinct species. Applied to the speciation process, the adaptive zones hypothesis makes two inter-related predictions: (i) large shifts between zones are relatively rare, (ii) when large shifts do occur they generate stronger RI than shifts within zones. Here we use ecological, phylogenetic, and behavioural data to test these predictions in *Timema* stick insects. We show that host use in *Timema* is dominated by moderate shifts within the systematic divisions of flowering plants and conifers, with only a few extreme shifts between these divisions. However, when extreme shifts occur they generate greater RI than do more moderate shifts. Our results support the adaptive zones model, and suggest that the net contribution of ecological shifts to diversification is dependent on both their magnitude and frequency. We discuss the generality of our findings in light of emerging evidence from diverse taxa that the evolution of RI is not always the only factor determining the origin of species diversity.

Keywords: host preference, phylogenetics, speciation, reproductive isolation, *Timema* stick insects

Introduction

A number of evolutionary models, such as Simpson's adaptive zones model of quantum evolution [1, 2], propose that small evolutionary changes within adaptive zones (i.e., changes within a broad resource or habitat category, *sensu* [3, 4]) are common, but that more extreme evolutionary change occurs rarely [5, 6]. Most evidence for such models stems from deep macro-

63   evolutionary timescales and high taxonomic levels, such as orders or families [1, 2, 5]. Thus, the

64   processes and mechanisms generating these patterns are not well understood. For example, the

65   roles of random drift, fluctuating selection, and macro-mutation (e.g., 'hopeful monsters'[7]) in

66   generating patterns consistent with these models remain unclear, but must be resolved to

67   understand whether and which micro-evolutionary processes best explain broad scale macro-

68   evolutionary patterns.

69

70   Here, we specifically apply the adaptive zones model to the speciation process, which often

71   occurs by populations diverging into partially reproductively isolated ecotypes or subspecies, and

72   eventually into strongly reproductively isolated species [8-15]. Such a differentiation process or

73   'speciation continuum' has been observed in fish [16-19], amphibians [20], birds [21], plants

74   [22], and insects [13, 14, 23-25]. However, it is often unclear why populations differ in levels of

75   RI [9-11, 26]. How such differences affect the diversification of a clade is then further

76   complicated by the relative frequencies with which different levels of RI are reached. The

77   adaptive zones hypothesis can be applied to explain this variation, making two inter-related

78   predictions: (i) shifts between zones are relatively rare, (ii) when large shifts between zones do

79   occur they generate stronger RI than shifts within zones (Figure 1).

80

81   Note that even without invoking the adaptive zones model *per se*, these predictions should hold;

82   large ecological shifts that generate strong RI may be rare. Moreover, although we here test the

83   adaptive zones model using discrete categories of ecological divergence, similar logic could be

84   applied to continuous scenarios. Just as one may ask whether shifts between more extreme

85   categories generate more RI, one could test whether more extreme quantitative shifts in ecology

86   (e.g., temperature, elevation, aridity) generate more RI. For example, it has been shown that

87   more extreme differentiation in quantitative ecological variables is associated with stronger RI

88   across disparate plant and animal taxa, although this work did not consider phylogenetic shifts

89   *per se* [27, 28].

90   Testing these predictions is challenging because it requires integration of macro-evolutionary

91   patterns, for example at phylogenetic time-scales, with data on micro-evolutionary processes and

92   the evolution of RI. Most generally, such studies might help connect broad diversification

93   patterns (i.e., defined as the net result of the speciation and extinction processes over time) to

94   micro-evolutionary processes. We provide such a study here by integrating phylogenetically-

95   based inferences on rates of host shifts for >100 host-associated populations of 11 *Timema* stick

96   insect species with experimental estimates of host-plant preference. Because *Timema* feed, mate,

97   and spend most of their lives on their hosts [29, 30], host preferences are likely to translate to

98   premating isolation in nature. Thus, we here use results from host preference experiments in the

99   lab as a proxy for RI, with the understanding that future work testing RI in nature is warranted.

100   Notably, *Timema* feed on a very wide range of hosts, [29, 30] but the frequency of host shifts of

101   different magnitude over the ~30 million year old history of this group has yet to be quantified

102   [25].

103

104   We thus here study *Timema* taxa that use a wide range of conifer (e.g., pine, cedar, redwood, and

105   fir) and flowering plant (e.g., oak, roses, and manzanita) hosts (Figure 2, Table S1; a host-plant

106   population is defined as conspecific individuals collected from a common host genus at a

107   geographic locality). In this context, we consider conifer and flowering plant hosts to represent

108   different adaptive zones and thus shifts between them to be large relative to those within plant

109   divisions, based on: (i) the fact that few insect species (or even sets of closely-related species)

110   use both these plant divisions as hosts [31], and (ii) the deep phylogenetic divergence between

111   these two divisions and their great differences in chemistry, physical structure, and evolutionary

112   dynamics [32-36].

113

114   We first used phylogenetic information and host plant use to infer the frequency of shifts

115   between conifer and flowering plant hosts [25], relative to shifts between hosts within each

116   division. An adaptive zones model would be supported by host shifts overall being common, but

117   those between conifers and flowering plants being rare. Second, we tested for an association

118   between the magnitude of a host shift (i.e., within or between plant divisions) and divergence in

119   host preference, a form of premating RI for insects such as *Timema* that mate on their host plants

120   [37-39]. Our results support the adaptive zones model, and suggest that the net contribution of

121   ecological shifts to RI can depend on the shifts' magnitude. When larger shifts occur less often,

122   their rarity increases waiting times to speciation. Thus, our findings add to emerging evidence

123   that although the evolution of RI is a key component of the speciation process, it may not always

124   be the factor controlling the frequency at which new lineages originate [40-42].

125

126   **Methods and Materials**

127

128   *Analysis of transition rates between hosts*

129

130   Our sampling effort covered regions where *Timema* have been systematically studied over the

131   last two decades [25, 43], and searches were done of the known common hosts of each species.

132   Missing host taxa would be problematic for our study only if this sampling was not random (i.e.

133   systematically missing populations on conifers), which is unlikely. Details of the populations

134   studied here are contained in [25] and in Table S1.

135

136   We first tested whether shifts between conifers and flowering plants occurred multiple times. We

137   used the reduced-representation sequence data from the 57 geographic populations previously

138   studied in [25] to infer phylogenetic relationships among *Timema* species and populations. This

139   was done using data deposited in a Dryad repository (https://doi.org/10.5061/dryad.nq67q;

140   linkage-group multiple alignments under the section "Phylogenetic inference and molecular

141   dating") to produce two new multiple alignments: selecting only the sites with at least two

142   different nucleotides ('strict-ASC'; 5797 variable sites), which allows using ascertainment bias

143   models for inferences, and selecting also the sites with at least one ambiguity ('relaxed'; 19,556

144   variable sites). We used IQTREE 1.6.2 [44] to carry out automatic substitution and partitioning

145   model selection and to infer maximum-likelihood trees using topological constraints in order to

146   test five different hypotheses: clustering by host plant division ('division', implies a single shift

147   between conifers and flowering plants), clustering by *Timema* species ('species', allow for

148   multiple shifts within species), clustering by *Timema* species and host plant division within

149   *Timema* species ('division within species', allows a single shift within each species), using the

150   previous Bayesian inference from [25]('BEAST'), and a maximum-likelihood tree inferred with

151   IQTREE for this study ('free', no topological constraint whatsoever). Then, we estimated site-

152   wise log-likelihoods and performed Shimodaira-Hasegawa (SH, [45]), weighted Shimodaira-

153   Hasegawa (WSH,[45]) and Approximately Unbiased (AU,[46]) tests using IQTREE and consel

154   1.20 [47].

155

156   Subsequently, we reconstructed ancestral states using the function rayDISC from the R package

157   corHMM 1.24 [48] in R 3.4.4 [49]. This function allows estimates of transition rates and

158   ancestral states for multistate traits given a tree, allowing for polymorphism on the tips (i.e.,

159   assigning equal likelihoods to several hosts for a given population in our case), and recognising

160   both gains and losses of host plant genera. First, we estimated ancestral states using the Bayesian

161   maximum credibility tree from [25], coding the hosts of each of the 57 geographic populations as

162   conifer, flowering plant, or both, and estimating the root probability with the method described

163   in [50, 51]. This allowed us to visualize ancestral state marginal probabilities on the nodes of the

164   tree (Figure 3).

165

166   Additionally, however, we were interested in comparing the transition rates between hosts in

167   different genera or families belonging to the same division ('within', i.e., conifer to conifer or

168   flowering plant to flowering plant) to those between different divisions ('between', i.e., conifer

169   to flowering plant or vice versa). Therefore, we used the host genera as states and fit five

170   different models (Figure 4): (1) r1: all transition rates forced to have the same rate, (2) r2: one

171   rate from transitions between conifers and flowering plants and vice versa, and another rate for

172   transitions within conifers or within flowering plants; (3) r3a: one rate for transition within either

173   conifers or flowering plants, one rate for transitions from conifer to flowering plant, and another

174   rate for flowering plant to conifer; (4) r3b: one rate for transitions within conifers, one rate for

175   transitions within flowering plants, and another rate for transitions between conifers and

176   flowering plants and vice versa; and (5) r4: one rate for transitions from conifer to flowering

177   plant, another rate for flowering plant to conifer, another rate for transitions within conifers, and

178   another rate for within flowering plants.

179

180   To assess the robustness of our results, we used five priors for the root: same probability for all

181   host genera ('flat'), root probabilities weighted using estimated transition rates following [52]

182   ('yang') or [50, 51] ('madd'), same probability for all conifer host genera ('con'), and same

183   probability for all flowering plant genera ('flo').

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184

185   The results were similar in most of the cases, but we focus our description of the results on the

186   inferences using the method of [50, 51]. We ran the analyses on 1000 trees taken randomly from

187   the posterior distribution of time calibrated trees (from [25]) to account for phylogenetic

188   uncertainty. Rates and states were estimated jointly (node.states="joint", state.recon="estimate"),

189   because such an approach is less prone to getting stuck in local optima [53]. However, most

190   other studies commonly carry out marginal reconstructions (i.e., rate inference followed by

191   ancestral states estimation), and we also did that here for comparative purposes

192   (node.states="marginal", state.recon="subsequently").

193

194   *Host preference trials*

195

196   We carried out host preference experiments with 3492 individuals from 70 populations (35 pre-

197   determined population/species pairs; see Table S2 for details of species, populations, sample

198   sizes, hosts tested, etc.). Tested individuals were captured with sweep nets and placed in plastic

199   cups containing cuttings of two plant species: (1) the plant species that individual was collected

200   on ('native host') and (2) a different plant species, usually the plant species used by the alternate

201   paired population ('alternative host'). In the morning, the plant species that each individual was

202   found to be resting on after overnight incubation was recorded as the preferred plant species.

203   Each individual was used only once and trials where individuals did not choose a host were

204   excluded from analysis. We quantified host preference differences between paired populations

205   using different host plants in nature (Table S2).

206

207   *Host preference differentiation as a function of host-plant use*

208

209   The goal was to test if population pairs on more phylogenetically distant hosts (i.e., conifers

210   versus flowering plants) exhibited greater divergence in host preferences than those using more

211   similar hosts. The pairs were chosen primarily to represent a range of divergence in host plant

212   use, including pairs using the same genus, different genera in the same plant division, and

213   different plant divisions. In addition, the taxa compared were generally not distantly related to

214   one another, encompassing also the practical component of access to taxon pairs across disparate

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215   parts of the widespread species ranges. Accordingly, 24 pairs were analysed (the remainder used

216   the same host, and were thus not relevant here, but were used for tests of phylogenetic

217   conservatism using individual populations described below). These pairs represented both those

218   where one population used a conifer host and one used a flowering plant host (n = 8 pairs, mean

219   number of individuals tested per population = 37) and those using two different flowering plants

220   hosts (n = 16 pairs, mean number of individuals tested per population = 46).

221

222   Mean preference for individual populations was calculated as the proportion of trials that one of

223   the offered hosts was chosen, a value that ranges from zero (focal host never chosen) to one

224   (focal host always chosen). We then calculated host preference divergence between pairs as the

225   absolute difference in mean preference between pairs, a value that also ranges from zero

226   (identical preferences of the two populations) to one (completely divergent preferences between

227   the two populations, i.e., 100% preference divergence). Note that this value of host preference

228   divergence is identical when either of the two offered hosts is used to calculate mean preference

229   for individual populations. Phylogenetic distance between hosts was grouped into two categories:

230   moderate for the pairs on two different flowering plants and strong for the pairs on a conifer

231   versus a flowering plant host (see Introduction for justification of these categories).

232

233   Because preference divergence was bounded between zero and one we employed beta regression

234   to model the influence of divergence in host plant use on divergence in host plant preference

235   (dependent variable) using the betareg function in the package betareg 3.1-2 [54] in R, specifying

236   "logit" as link function. We performed analyses with the complete data set of 24 comparisons,

237   and two subsets including either only conspecific population pairs (n = 18 pairs), or only

238   conspecific population pairs from the same geographical site (n = 13 pairs). We obtained

239   congruent results from all three analyses (see Results).

240

241   *Phylogenetic conservatism of host preference*

242

243   Phylogenetic relatedness of taxa generates non-independence of data points obtained from

244   multiple populations or species. To assess the need to account for this effect in the analysis of

245   host preference in *Timema*, we tested for the presence of phylogenetic signal in the strength of

246   the preference for the native host. We analysed the host preference data described above (see

247   also Table S2) in combination with the population level Bayesian time-calibrated maximum

248   credibility tree from [25] pruned to represent the 48 populations for which host preference data

249   was available (corresponding to 28 geographic localities on the tree). Host preference of each of

250   these individual populations was estimated as the proportion of trials the native host was picked

251   over the alternative host. In cases where a population with host preference data was not

252   represented in our phylogeny (n = 25), we chose the geographically closest population of the

253   same species as its representative in the phylogeny (mean distance to the nearest population was

254   not overly large, being 17.7 km).

255

256   As several populations with host preference data mapped to the same tip in the phylogeny, we

257   used two approaches to assign trait values to those tips: (1) we used the mean of host preference

258   for populations mapping to the same tip, and (2) for each tip represented by multiple populations

259   we sampled a trait value randomly from those populations 1000 times to generate a sample

260   distribution of host preference for those populations. Results were congruent between the two

261   approaches (see Results below). We used the function phylosig in the package phytools 0.6-99

262   [55] in R to calculate the K statistic of [56] and its statistical significance. Higher values of K

263   indicate successively stronger phylogenetic signal, with K=0 in the absence of phylogenetic

264   signal, K=1 under a Brownian motion model of trait evolution, and K>1 when trait evolution is

265   more constrained. In general, we found a lack of phylogenetic signal for host preference (details

266   below).

267

268   **Results**

269

270   *Frequency and magnitude of host shifts*

271

272   We found that large ecological shifts between conifer and flowering plant hosts have occurred

273   multiple times among our study populations, including shifts within species (Table S3,

274   Approximately Unbiased test, p < 0.001 for both clustering by host divisions, and clustering by

275   host divisions within *Timema* species). Indeed, host shifts in general appear common in *Timema,*

276   likely facilitated by standing genetic variation in the ability to use novel hosts [30].

277

278   Phylogenetic analyses of transition rates between hosts support a key prediction of the adaptive

279   zones model; i.e., that large host shifts between conifer and flowering plant hosts are relatively

280   rare. Specifically, we compared the fit of five transition rate models by maximum likelihood

281   (Figure 4). Our main interest was on the support for a 'one-rate model' that enforced a single

282   transition rate irrespective of whether shifts were within or between plant divisions, relative to

283   models that allowed rates to vary between different types and magnitudes of host shifts. Our

284   analyses revealed that the one-rate model was consistently the least supported one (Figure 5,

285   Table S4, difference in Akaike Information Criterion values corrected for sample size (ΔAICc)).

286   The best-fit model allowed for transitions within each division and between them ('three-rate

287   model b'). However, the most noticeable increase in support was observed when moving from

288   the one-rate model to a two-rate model that allowed the rates for transitions between divisions to

289   differ from those within divisions (Figure 5). Notably, these results were robust to using multiple

290   root probabilities and inference methods (Figure S1, Table S4). Thus, we found that transition

291   rates within divisions were ~5x higher than those between divisions (Figure 5, range ~3-10x

292   higher using different inference methods and root probabilities, Figure S2-S3, Table S5).

293

294   *The evolution of premating isolation due to host preference*

295

296   Next, we were interested in the evolutionary consequences of extreme host shifts. For 24

297   population pairs (2252 trials) that use different hosts, we found that host preference was ~2-3x

298   more differentiated between *Timema* taxa feeding on different divisions than between those

299   feeding on different families within divisions (Figure 6). This result was robust to whether we

300   considered all 24 taxon-pairs ($z = 3.50$, $p = 0.0005$, beta regression; Table S6), the 18 within-

301   species comparisons ($z = 2.72$, $p = 0.0065$), or the 13 within-species within-locality comparisons

302   ($z = 2.49$, $p = 0.0128$).

303

304   *Phylogenetic conservatism of host preference*

305

306   We found little to no evidence for phylogenetic conservatism of host preference, justifying the

307   population level analyses above. Specifically, Blomberg's K was found to be low and non-

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308 significant in the core analysis using the mean as the trait value for tips represented by more than

309 one population (K = 0.101, p = 0.747). This result was robust to the approach of considering the

310 mean, because randomizing the trait value to be equal to that from one of the populations when

311 multiple populations represented one tip always resulted in K's smaller than 0.2. Furthermore,

312 only seven out of 1000 permutations yielded a P-value lower than 0.05, all being non-significant

313 after correction for multiple comparisons.

314

315 **Discussion**

316

317 We use ecological and behavioural data in a phylogenetic comparative framework to test general

318 predictions about adaptive zones and the speciation process. Although *Timema* stick insects use a

319 wide breadth of host plants, we found that host use is dominated by moderate shifts between

320 families within flowering plants or conifers, with only a few extreme shifts between these plant

321 divisions. When extreme shifts do occur, however, they likely generate greater premating

322 isolation (via host preference) than do moderate shifts (Figure 1). These results are consistent

323 with the adaptive zones model and suggest that the net contribution of ecological shifts to

324 diversification can reflect a balance between their magnitude and frequency.

325

326 As in many correlational or comparative studies, which abound in evolutionary biology,

327 causation is difficult to definitively infer. Thus, it is possible that host preference itself affects the

328 frequency of extreme shifts. However, we consider it more likely that preference evolution is a

329 consequence (rather than cause) of extreme shifts because: (1) host preferences are generally

330 quite modest in absolute terms such that they are unlikely to strongly constrain host shifts

331 (Figure 6; Table S2), and (2) they appear evolutionarily labile, with no evidence for phylogenetic

332 conservatism. Below we discuss the causes of observed patterns of host shift, the completion of

333 speciation, and limits to diversification (i.e. the net result of the speciation and extinction

334 processes over time). Future work could usefully consider whether this pattern applies to other

335 forms of RI.

336

337 *Causes of observed patterns of host shift*

338

339   We have shown that several large ecological shifts between conifers and flowering plants have

340   occurred during the diversification of *Timema*, although their frequency is much lower than

341   shifts within the divisions. There are at least two core factors that could limit the frequency of

342   shifts between evolutionary distant host plants. First, there could be inherent adaptive

343   constraints, as highly different host plants are likely to constitute distant adaptive peaks. For

344   example, specialization can involve trade-offs resulting in metabolic constraints, in turn making

345   shifts to new hosts more difficult [57, 58]. Performance experiments indicate that this is not

346   strongly the case in *Timema* in terms of the physiological ability to digest new hosts [30], but the

347   existence of trade-offs associated with crypsis and predation is likely [59, 60].

348

349   Second, the geographic distribution of the plants can put constraints on the colonization of new

350   hosts. Opportunities to shift between conifers and flowering plants may have been ample for

351   *Timema*, as both kinds of host plants are found commonly intermixed throughout California

352   currently, and were so during most of the *Timema* diversification history [61, 62]. Nonetheless,

353   this geographic overlap has not been formally quantified for the populations studied here. Further

354   work is thus required to quantify the contribution of inherent biological constraints versus the

355   geographic arrangement of plants on the host shifts, but either way shifts between divisions are

356   rare. Future insights on the role of syntopy of host plants would need to consider their past

357   distributions over long time periods, and at a fine geographical scale. One methodological

358   consideration is that most of *Timema* species not included in our analysis feed on conifers (i.e.,

359   those outside of California)[29, 63-65]. If most of these constitute a sister group to Californian

360   species, our results would hold valid, but could be limited to the Californian lineage.

361

362   Interestingly, when each plant division is considered, transition rates between conifers were

363   higher than rates between flowering plants. Gymnosperms are known to have lower

364   morphological and chemical diversity than angiosperms, as well as lower morphological and

365   genomic evolutionary rates [31, 66, 67]. This could translate into different conifers representing

366   relatively closer adaptive peaks when compared to flowering plants, thus making shifts between

367   them easier. In addition, mixed conifer forests are common in California, but tend to be restricted

368   to particular altitudinal bands and separated in geographic space [67-69], which may have

369   favoured repeated parallel shifts between conifers. Lastly, we cannot discard a potential effect

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370 derived from our choice of taxonomic level (i.e. genus). For example, for most of the conifer

371 genera that *Timema* use, the use is restricted to a single species, whereas for flowering plant

372 genera usually there are several species per genus used [30, 63]. This could result in reduced

373 transition rates within flowering plants. In other words, our conclusions hold well for transition

374 rates between genera, and further work on transitions between species is warranted.

375

376 *Evolution of host preferences, premating isolation, and completing speciation*

377

378 Studies of *T. cristinae* have shown that host preferences are likely partially heritable, with

379 'hybrids' between host-plant ecotypes exhibiting preferences intermediate between the parental

380 forms [70-72]. However, further work is required to determine the relative contribution of

381 genetic versus induced environmental factors to this form of RI during the diversification of

382 *Timema*, and to RI in general across taxa. Induced effects on RI have been reported for

383 imprinting of song in birds [73], cultural differences among killer whale ecotypes [74], and host

384 or mate preference in other insects [75, 76]. On the other hand, if environmental effects can be

385 reversed, this could decrease RI. Further work on the role of genetic and environmental effects in

386 speciation is warranted.

387

388 In contrast with previous work on patterns of host use in nature [29], we did not find evidence

389 for phylogenetic conservatism for behavioural host preference. This is likely because

390 phylogenetic relations in past work were based on a single marker (mitochondrial DNA) and not

391 as accurate as those used here, and because host use in nature doesn't necessarily correspond to

392 behavioural host preference (i.e., less preferred hosts may be used in nature due to availability,

393 necessity, or convenience)[71]. Moreover, our results are in agreement with recent experiments

394 showing that most populations retain plasticity in host use [30].

395

396 Finally, we note that even the most extreme host preferences documented here were not perfectly

397 divergent between any of the tested taxon pairs (i.e., we never observed a 100% difference

398 between a pair). Thus, RI due to host preference does not appear to reach completion. In part,

399 this could reflect that our experimental design in the lab under-estimates host preferences in

400 nature, but even so it seems unlikely that host preferences alone can complete speciation in

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401   *Timema.* Moreover, the frequency of shifts between very different hosts is very low such that

402   they alone are unlikely to explain late stages of speciation and the diversification of *Timema*.

403   Thus, the completion of speciation likely requires other factors, such as periods of geographic

404   isolation and restricted gene flow [25], and the evolution of additional forms of RI. Indeed, there

405   is evidence for RI due to chemical-mediated mate choice [25, 72, 77-79], selection against

406   immigrants onto new hosts and hybrids [59, 60, 77, 79], and postmating, prezygotic isolation

407   [72, 80]. Further work is required to test how moderate versus extreme host shifts affect these

408   forms of RI, if they do at all.

409

410   *Limits to the rate of speciation*

411

412   The evolution of RI is generally thought to be a key component of the speciation process [9, 81-

413   85]. However, several recent studies suggest that the evolution of RI is not the step limiting

414   speciation rates, particularly over long timescales. For example, the rate of the evolution of RI in

415   birds and flies, estimated experimentally, is uncoupled from speciation rates estimated using

416   phylogenies [42]. Likewise, the diversification of Himalayan songbirds appears limited by the

417   rate of niche filling, not the acquisition of RI [40]. In insects, host shifts usually result in an

418   increase of RI and can initiate speciation processes, but their relative contribution to insect

419   diversification is unclear [3, 38, 57, 86, 87].

420

421   Our results inform this issue by showing how a key factor other than RI, i.e., the rate at which

422   new niches are colonised, can be important for understanding diversification. In particular,

423   ecological shifts large enough to generate substantial RI may be rare. Thus, the total contribution

424   of an ecological shift of particular magnitude to the diversification of a clade might be the net

425   result of the amount of RI it confers and its frequency. These two factors might be opposing and

426   are potentially interlinked, and consequently the empirical role of ecological shifts in speciation

427   requires further work. Further studies that examine a range of closely related taxa that vary both

428   in RI and the magnitude of ecological shifts they underwent are warranted.

429

430   *Conclusions*

431

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432 In summary, our results provide evidence that the ecological magnitude of a host shift can affect

433 levels of RI. Shifts themselves appear common in *Timema* and this is likely facilitated by

434 standing genetic variation in the ability to use novel hosts [29, 30], which is likely maintained at

435 least in part by gene flow [72, 79, 88, 89] and balancing selection [43, 90, 91]. Finally, the

436 results inform limits to divergence, as they show that shifts between ecologically distant host

437 plants are rare, and therefore unlikely to explain diversification on their own. Thus, the rate of

438 species formation could largely be the result of the waiting time for shifts between distant

439 adaptive peaks coupled with events that create geographic isolation.

440

441 **Data accessibility**

442

443 The data reported in this paper, including custom code written for analyses, have been archived

444 in Dryad Digital Repository XXXX (number can only be provided by Dryad *after* the

445 manuscript is accepted). Code used for the analyses have been deposited in bitbucket repository

446 https://bitbucket.org/visoca/eco_shifts_timema.

447 **Author's contributions**

448 MM and VS carried out phylogenetic hypothesis testing, transition rate inferences and

449 phylogenetic conservatism analysis. PN performed the feeding preference trials. MM and ZG

450 carried out the host-preference differentiation analysis. MM, VS and PN participated in the

451 design of the study and drafted the manuscript. All authors critically revised the manuscript, gave

452 final approval for publication and agree to be held accountable for the work performed therein.

453

454 **Competing interests**

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456 We have no competing interests.

457

458 **Funding**

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472   **Materials and correspondence:** patrik.nosil@cefe.cnrs.fr

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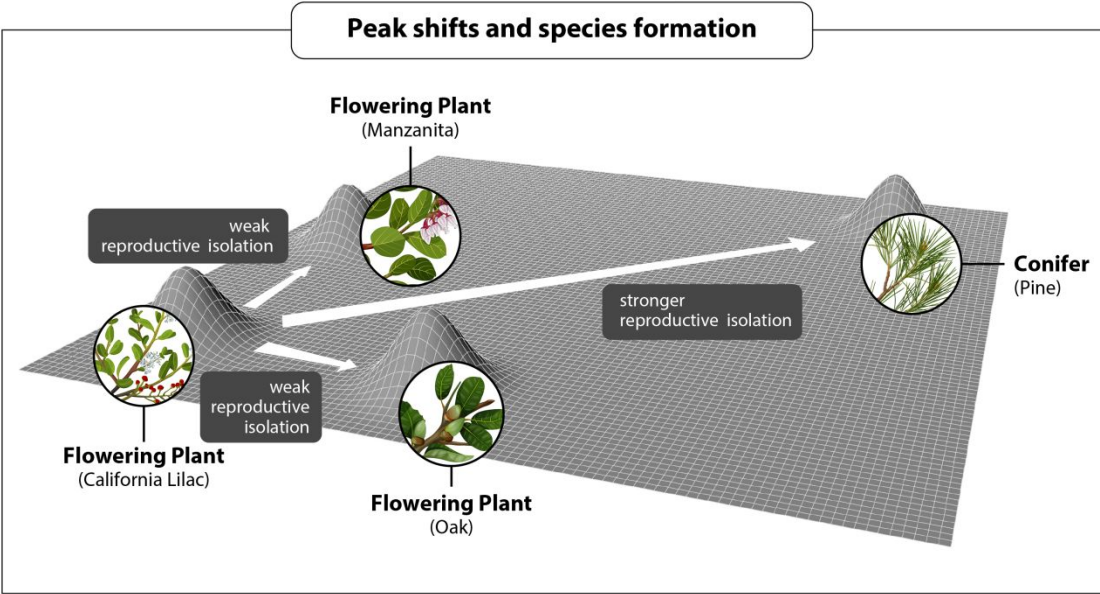**Peak shifts and species formation**

474

475   **Figure 1. Ecological shifts and the process of speciation.** A schematic depiction of how large

476   peak shifts between flowering plant and conifer hosts, although relatively rare, generate greater

477   RI than more moderate host shifts among flowering plant families.

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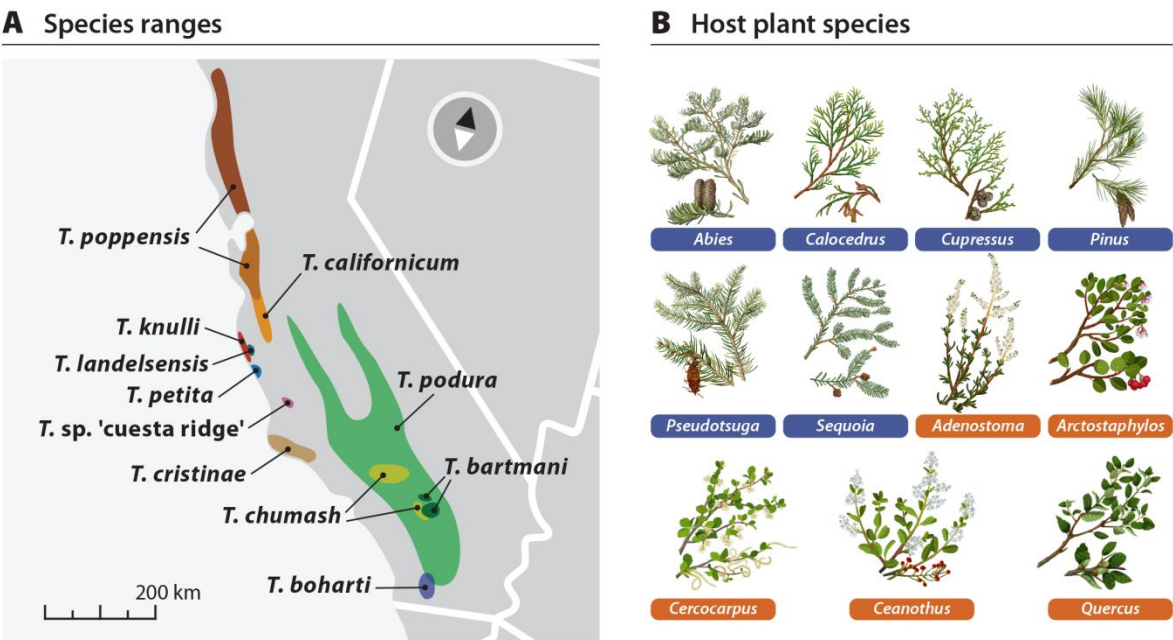Submitted to Phil. Trans. R. Soc. B - Issue

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Figure 2. *Timema* species and populations, and host-plant use. (A) *Timema* species ranges (from [25]). (B) Host-plant species used by *Timema* populations used in this study (conifers have blue labels, flowering plants have orange labels).

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Timema species, phylogenetic tree and host plant use, including ancestral use inferences
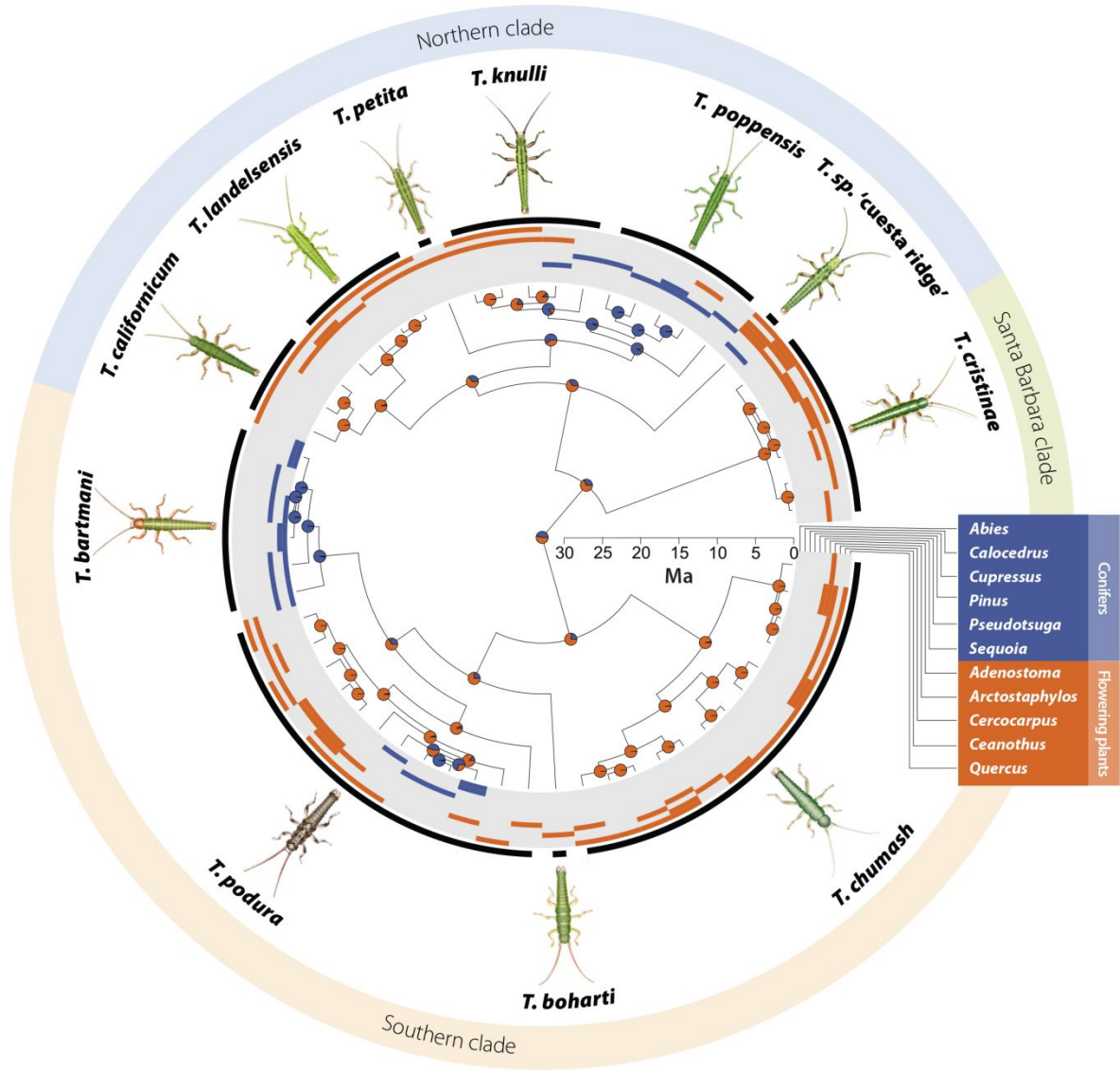
Figure 3. *Timema* **species phylogenetic relationships and host plant use, including ancestral use inferences using conifers and flowering plants as states.** The bars on the periphery depict the host-plant use of each population. The pie charts on the internal nodes represent the proportional likelihoods of conifers (blue) and flowering plants (orange) for reconstructed ancestral states.

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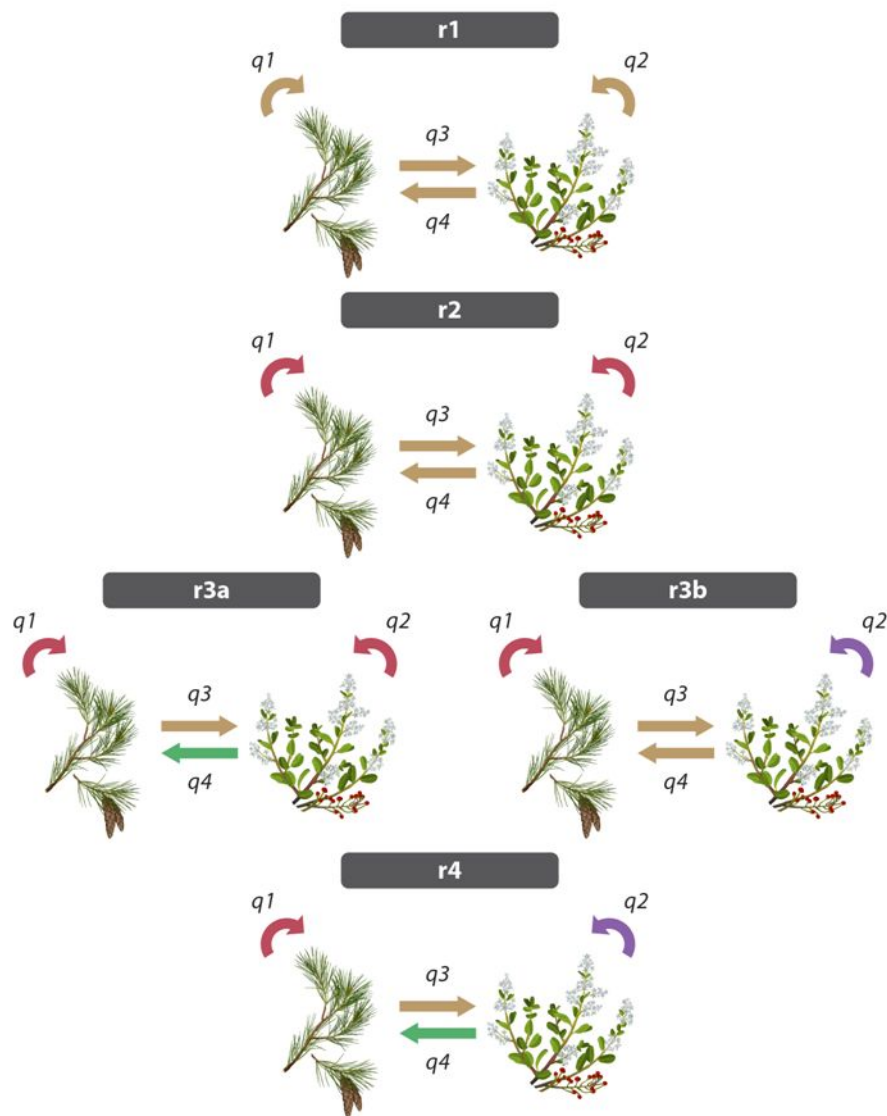
Transition rate models

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Figure 4. The models tested in transition rate analyses. Graphical representation of the

transition rate models built considering four rates: from conifer to conifer ($q1$), from flowering

plant to flowering plant ($q2$), from conifer to flowering plant ($q3$), and from flowering plant to

conifer ($q4$). The one-rate model (r1) assumes a single rate for all shifts ($q1=q2=q3=q4$). The

two-rate model (r2) assumes a single rate for within-division shifts and another one for between-

division shifts ($q1=q2{\neq}q3=q4$). The three-rate model 'a' (r3a) assumes a single rate within

divisions, and different rates for shifts from conifers to flowering plants and vice versa

($q1=q2{\neq}q3{\neq}q4$). The three-rate model 'b' (r3b) assumes a single rate for between-division shifts,

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499   and different rates within conifers and within flowering plants ($q1{\neq}q2{\neq}q3{=}q4$). The four-rate

500   model (r4) assumes different rates for all kinds of shifts ($q1{\neq}q2{\neq}q3{\neq}q4$).

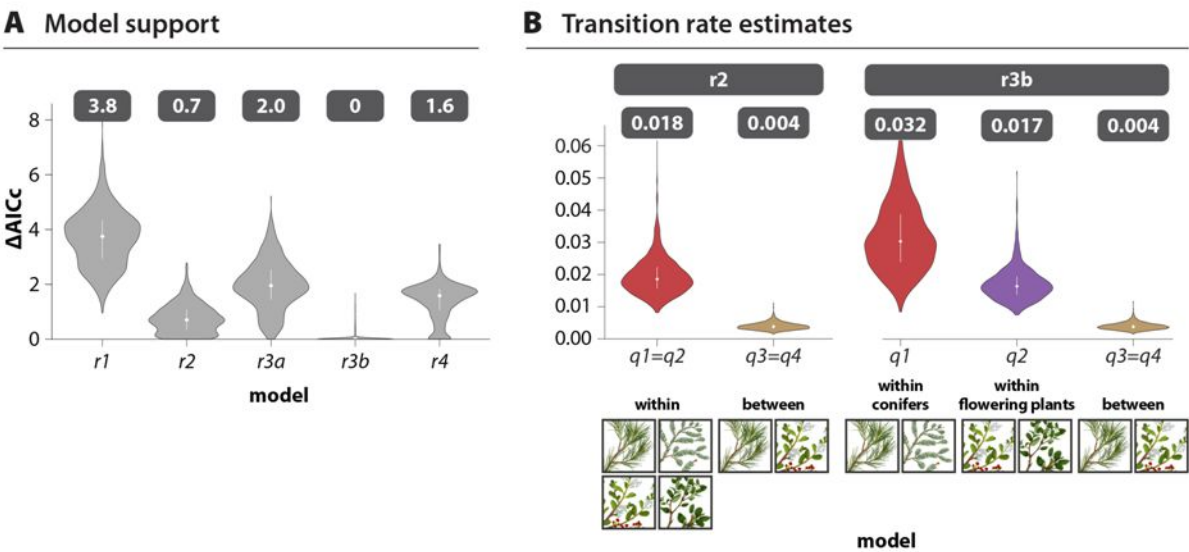Page 23 of 29

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Figure 5. Support for each transition-rate model (see also Figure 3). (A) Violin plots depict the distribution of ΔAICc values (difference in sample-size corrected AIC between the focal model and the best model) after fitting the models by maximum likelihood to 1000 trees randomly taken from the Bayesian posterior distribution of trees from [25]. The median ΔAICc for each model is shown above each violin plot. (B) Distribution of transition rate estimates for the most supported models r2 and r3b. Colours of violin plots follow the same scheme as rates in Figure 3. The median of each rate is shown above each violin plot. The white diamonds and bars represent the median and the interquartile range, respectively.
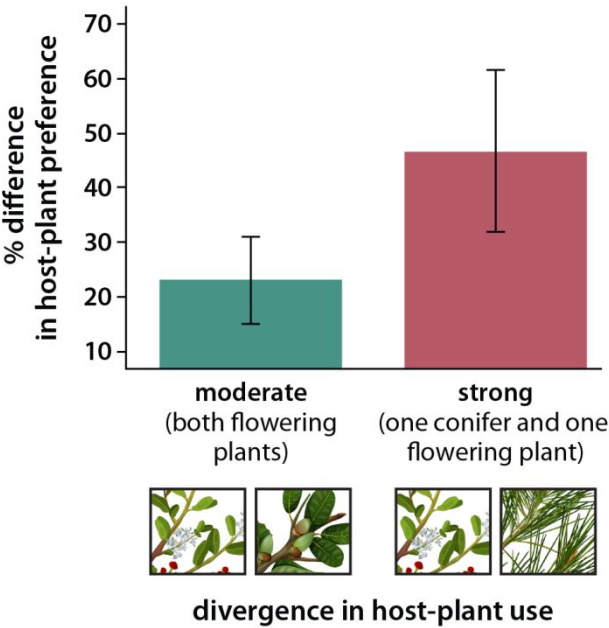
Figure 6. Behavioural host preference experiments. Shown is mean divergence in host preference (±95% CI) between population pairs differing in the degree to which their hosts differ.

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